Molecular Detection of *Mycoplasma haemofelis* and 'Candidatus *Mycoplasma haemominutum*' Infection in Cats by Direct PCR Using Whole Blood without DNA Extraction

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(Received 27 November 2007/Accepted 20 May 2008)

ABSTRACT. Detection of hemotropic *Mycoplasma* spp. infection was attempted in cats by PCR using whole blood without DNA extraction. A total 46 of 54 (85%) cats with suspected *Mycoplasma* spp. infection showed a positive reaction, corresponding completely with the results of standard PCR testing. The direct PCR assay was sensitive enough to detect more than 0.0061% parasitemia for 'C. M. haemominutum' and 0.0075% parasitemia for *M. haemofelis*. These data indicate that the direct PCR assay might be sufficient for use as a tool in clinical examinations.

KEY WORDS: blood, feline, hemoplasma.

J. Vet. Med. Sci. 70(10): 1095–1099, 2008

Mycoplasma (*M.*) *haemofelis*, 'Candidatus (*C.*) *Mycoplasma haemominutum*' and 'C. *Mycoplasma turicensis*' are minute, gram-negative, epicellular bacteria infecting the feline erythrocyte and causing hemolytic anemia, thrombocytopenia, fever and jaundice [12, 16]. Definitive diagnosis of hemotropic *Mycoplasma* spp. infection is made by examination of a thin Wright-Giemsa-stained blood smear, but this method is unreliable because the tiny organisms often resemble stain debris, protein precipitates and Howell-Jolly bodies. Furthermore, parasitemic episodes are recurrent, and so examination under a microscope sometimes cannot detect the organism in some clinical cases. Recently, some reports have indicated that molecular detection of *Mycoplasma* spp. infection using polymerase chain reaction (PCR) based on the 16S rRNA gene of hemotropic *Mycoplasma* spp. is more sensitive and specific than cytological examination [5, 7, 8, 15]. Therefore, PCR analysis has come to prevail as a useful and sensitive examination in veterinary laboratories in Japan. However, the cost of examination is high, and the time needed is not short. The complicated procedure and time required for the PCR assay are suspected as being the main reasons for these problems. So, it is important that these cost and time problems will be resolved in order to extend the PCR assay further into the veterinary field. A highly capable Taq polymerase has been developed that enables more efficient amplification of DNA [4]. Furthermore, several reports have revealed that direct PCR using a template such as whole blood and feces is able to detect various gene abnormalities and infectious diseases [3, 10, 11]. It is necessary to confirm the capacity of direct PCR to detect hemotropic *Mycoplasma* spp. infection by

analyzing a specific population. Both the *M. haemofelis* and 'C. *Mycoplasma haemominutum*' strains were widespread in domestic cats in Japan, but sole infection with 'Candidatus *Mycoplasma turicensis*' has not been confirmed [6]. Therefore, direct PCR analysis to detect *M. haemofelis* and 'C. *Mycoplasma haemominutum*', was carried out in a total of 59 blood samples derived from cats with a suspected *Mycoplasma* spp. infection and healthy cats, and the results were compared with the standard method described previously [15].

A total of 59 blood specimens from 54 cats with a suspected *Mycoplasma* spp. infection, based on clinical signs and haematological abnormalities, and 5 healthy cats were used in this study. The hemotropic *Mycoplasma* spp.-infected cats were characterized by the presence of anemia (PCV of less than 28%), parasitemia in a blood smear and clinical features including fever, splenomegaly, jaundice and hemoglobinuria. Peripheral blood anticoagulated with EDTA was obtained from the Veterinary Teaching Hospital of Azabu University and other veterinary clinics. Parasitemia was estimated by counting the number of parasite-infected erythrocytes per 1,000 cells on blood films stained with Wright-Giemsa solution. Extraction of genomic DNA and PCR were performed following previously reported procedures [15]. Briefly, after addition of 500 μ g lysozyme (Sigma-Aldrich, MO, U.S.A.) to 150 μ l of blood and incubation for 1 hr at 37°C, 200 μ g proteinase K and 100 μ l of 10% [w/v] sodium dodecyl sulfate (SDS) solution were added; the mixture was then incubated for 10 min at 65°C. Following this, 100 μ l of 5 M NaCl solution and 160 μ l of 5% [w/v] hexadecyltrimethylammonium bromide (Sigma-Aldrich) were added, and the mixture was incubated for 10 min at 65°C. The crude DNA was then purified by phenol/chloroform extraction and ethanol precipitation, as reported previously [13]. Standard PCR was performed with 10 ng

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of genomic DNA, 0.4 μ M of each primer, 1x PCR buffer, 0.2 mM of each dNTP, 2.5 units of *Ampli*Taq Gold DNA polymerase (Applied Biosystems, Foster, CA, U.S.A.) and sufficient sterile water to adjust the volume to 50 μ l. The sense and reverse primers for *M. haemofelis* were OH-OK1 and 00CB-r1, respectively; those for '*C. M. haemominutum*' were CA-B2 and 00CB-r1, respectively. The primer design was the same as reported previously [15]. The sequences of the sense primers (OH-OK1 and CA-B2) were 5'-ATGCCCCTCTGTGGGGGATAGCCG-3' and 5'-CTGGGAAAGTAGAGCTTCGCGAGC-3', respectively, and the reverse primer (00CB-r1) sequence was 5'-ATGGTATTGCTCCATCAGACTTTTCG-3'. Direct PCR for hemotropic *Mycoplasma* spp. using feline whole blood without DNA extraction was performed. Instead of DNA, 1 μ l of whole blood was used with 0.4 μ M of each primer, 1x LA PCR buffer II (Mg²⁺-free), 0.2 mM each dNTP, 4.0 mM MgCl₂ and LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan). Standard PCR was performed using an initial denaturation step of 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58.4°C for 45 sec, and extension at 72°C for 45 sec. Feline glyceralde-

hyde-3-phosphate dehydrogenase (FG3PDH) gene (GenBank accession no. M33197) was amplified simultaneously as an internal control using the forward primer 5'-CCTTCTTGACCTACACTACAT-3' and reverse primer 5'-CCAAAGTTGTCATGGATGACC-3'. The optimized cycling conditions for the direct PCR were the same as for the PCR described above, except for annealing at 58.4°C for 3 min. PCR products were electrophoresed on 2% agarose gel containing 0.5 μ g/ml ethidium bromide (Sigma-Aldrich), and formation of 273- and/or 202- and 452-bp DNA bands was considered to be a positive result for infection of *M. haemofelis* and/or '*C. M. haemominutum*' and G3PDH gene amplification, respectively.

Anemia (PCV < 28%) was seen in 38 of the 54 cats (70.4%) with a range of PCV values between 9–44% (mean, 20.9 $\pm$ 9.53%; Table 1). Hemotropic *Mycoplasma* spp.-like organisms were detected in 45 cats (83.3%) by cytological examination of blood smears, and parasitemia ranged from 1.1% to 56%. As a preliminary assay, a total of 4 polymerases, including LA Taq, Ex Taq, rTaq (Takara Bio Inc., Otsu, Shiga, Japan) and *Ampli*Taq Gold were applied to the direct and standard PCRs to assess the ability of DNA poly-

Table 1. Results of direct PCR assay using *Mycoplasma* spp. infected, non-infected (with other diseases) and healthy cat blood

Case No.	PCV (%)	Parasitemia (%)	<i>M. haemofelis</i> Blood	<i>M. haemofelis</i> DNA	' <i>C. M. haemominutum</i> ' Blood	' <i>C. M. haemominutum</i> ' DNA	Case No.	PCV (%)	Parasitemia (%)	<i>M. haemofelis</i> Blood	<i>M. haemofelis</i> DNA	' <i>C. M. haemominutum</i> ' Blood	' <i>C. M. haemominutum</i> ' DNA
<i>M. haemofelis</i> -infected							32	24	5.3	–	–	+	+
1	13	26	+	+	–	–	33	33	3.3	–	–	+	+
2	14	47.7	+	+	–	–	34	32	5.5	–	–	+	+
3	9	30	+	+	–	–	35	16	2	–	–	+	+
4	33	20	+	+	–	–	36	35	6.1	–	–	+	+
5	13	15	+	+	–	–	37	12	4	–	–	+	+
6	10	20	+	+	–	–	38	44	7	–	–	+	+
7	11	7	+	+	–	–	39	43	1.5	–	–	+	+
8	15	15	+	+	–	–	40	9	5.5	–	–	+	+
9	16	4	+	+	–	–	Mix-infected						
10	9	56	+	+	–	–	41	17	10.8	+	+	+	+
11	17	10	+	+	–	–	42	26	7.5	+	+	+	+
12	24	17	+	+	–	–	43	16	9.1	+	+	+	+
13	22	3	+	+	–	–	44	30	6	+	+	+	+
14	28	13.5	+	+	–	–	45	17	7.5	+	+	+	+
15	25	3.5	+	+	–	–	46	10	2	+	+	+	+
16	31	5	+	+	–	–	Diseased cat without <i>Mycoplasma</i> spp. infection**						
17	33	9.6	+	+	–	–	47	26	ND	–	–	–	–
18	35	ND*	+	+	–	–	48	31	ND	–	–	–	–
19	30	7.5	+	+	–	–	49	28	ND	–	–	–	–
20	29	3	+	+	–	–	50	20	ND	–	–	–	–
21	12	5	+	+	–	–	51	30	ND	–	–	–	–
22	11	5	+	+	–	–	52	25	ND	–	–	–	–
' <i>C. M. haemominutum</i> '-infected							53	30	ND	–	–	–	–
23	11	1.2	–	–	+	+	54	26	ND	–	–	–	–
24	11	12.5	–	–	+	+	Healthy cat						
25	12	4	–	–	+	+	55	38	ND	–	–	–	–
26	16	6	–	–	+	+	56	46	ND	–	–	–	–
27	14	10	–	–	+	+	57	42	ND	–	–	–	–
28	19	2.5	–	–	+	+	58	39	ND	–	–	–	–
29	24	3	–	–	+	+	59	43	ND	–	–	–	–
30	25	5.9	–	–	+	+							
31	26	1.1	–	–	+	+							

+: Amplification positive. –: Negative when LA Taq DNA polymerase was used. * Not detected.

** With anemia, anorexia, chronic renal failure, fever, hematuria, hemolytic jaundice, lethargy and/or vomiting.

Fig. 1. 2% agarose gel electrophoresis of the amplified DNA products from '*C. M. haemominutum*'-infected and *M. haemofelis*-infected cat blood (Cases 24 and 7) using various DNA polymerases, such as LA Taq, Ex Taq, rTaq and *AmpliTaq* Gold. B, whole blood sample; D, genomic DNA sample.

Fig. 2. Detection of parasite DNA by direct PCR using peripheral blood from cats naturally infected with *M. haemofelis* and '*C. M. haemominutum*'. Analysis was performed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Cases 23 and 24, infected with '*C. M. haemominutum*', showed amplification of 202-bp DNA fragments from blood and DNA. Cases 1, 2 and 3, infected with *M. haemofelis*, showed amplification of a 273-bp DNA fragment. Case 41, infected with both *M. haemofelis* and '*C. M. haemominutum*', showed simultaneous amplification with both 273-bp and 202-bp DNA fragments. An uninfected control cat did not show a distinct band. The feline G3PDH gene was amplified as the internal PCR control marker, and samples showed a distinct or thin band. M, 100 bp ladder marker; B, whole blood sample; D, genomic DNA sample.

merase in the direct PCR method. The PCR conditions were the same as the direct PCR protocol described above, and the composition of the reagents was according to the instructions for each PCR kit. The whole blood and DNA of Cases 7 and 24 were used as a template. Amplification of a 273-bp DNA fragment indicated infection with *M. haemofe-*

lis, while amplification of a 202-bp DNA fragment indicated infection with '*C. M. haemominutum*'. The results of PCR analysis indicated that LA Taq and Ex Taq were able to amplify DNA fragments of both 16S rRNA genes of hemotropic *Mycoplasma* spp. and FG3PDH from whole blood, while rTaq and *AmpliTaq* Gold could not amplify the

Fig. 3. Sensitivity of direct PCR for detection of parasitic infection in cat whole blood. Whole blood from cats infected with the parasites was diluted serially 10-fold with normal cat blood, and direct PCR was performed using 1 μ l of each dilution. (A) '*C. M. haemominutum*' DNA was detected in a blood sample diluted at 10⁻³ (Case 36) with a parasitemia rate of 0.0061%. (B) *M. haemofelis* DNA was detected in a blood sample diluted at 10⁻³ (Case 19) with a parasitemia rate of 0.0075%. (C) A double infection of the above two parasites was detectable in a blood sample diluted at 10⁻³ (Case 42) with a parasitemia rate of 0.0075%.

16S rRNA and FG3PDH genes (Fig. 1). In regard to the standard PCR, all of these polymerases were able to amplify the DNA fragments of hemotropic *Mycoplasma* spp. and FG3PDH. Therefore, LA Taq and Ex Taq were considered to be appropriate polymerases for amplification of DNA fragments in direct PCR. Use of whole blood in PCR reactions results in a reddish or brownish color in the final PCR products. However, this does not interfere with detection of PCR products in 2% agarose gel electrophoresis.

A total 59 blood samples were used to detect hemotropic *Mycoplasma* spp. by direct PCR using LA Taq and by standard PCR using *Ampli*Taq Gold. The electrophoretic patterns of the PCR products and results of examination are shown in Fig. 2 and Table 1, respectively. In this study, amplification of the 273-bp DNA fragment was found in 22 cats (Cases 1–22), and amplification of the 202-bp DNA fragment was found in 18 cats (Cases 23–40). In addition, both the 202-bp and 273-bp DNA fragments were amplified

simultaneously in 6 cats (cases 41–46). Eight cats (cases 47–54) having other diseases, but not hemotropic *Mycoplasma* spp. infection, and 5 healthy cats were negative (Table 1). Thus, the DNA bands amplified by direct PCR were as visible as those of the standard method. The results of direct PCR corresponded to the standard PCR results completely. Even a blood-smear sample that was parasitemia-negative by cytological examination (case 18) was positive by direct PCR. The sequences of the PCR fragments amplified by direct PCR showed 100% homology with that for *M. haemofelis* or '*C. M. haemominutum*' (data not shown).

To determine the sensitivity of the direct PCR assay, blood samples from Case 36 (parasitemia: 6.1%), Case 19 (parasitemia: 7.5%) and Case 42 (parasitemia: 7.5%) were subjected to 10-fold serial dilutions (from 10⁻¹ to 10⁻⁴) using normal feline whole blood and were then amplified by direct PCR. Direct PCR was sensitive enough to detect parasite

genes for 'C. M. haemominutum' with 0.0061% parasitemia, *M. haemofelis* with 0.0075% parasitemia and double infection with 0.0075% parasitemia (Fig. 3).

In the present study, direct PCR using whole blood without extraction of DNA detected the parasite genes with the same degree of accuracy as standard PCR methods. PCR amplification using whole blood is expected to be a suitable method for detection of blood organisms; however, peripheral blood contains various PCR-inhibitory components, such as lactoferrin, hemoglobin and heparin [1]. Two polymerases, rTaq and *Ampli*Taq Gold, were unable to amplify the target genes in the direct PCR. Recently, the capabilities and sensitivities of DNA polymerases have been improved; consequently, Ex Taq and LA Taq have been suggested to be capable of more efficient amplification than the usual DNA polymerase [9]. LA Taq is known to be able not only to amplify a long length genome of more than 10-kb, but also to promote specific and appropriate amplification because it prevents incorporation of base pairs. LA Taq acts to proof reading activity (3'→5' exonuclease activity), so it is possible that higher efficiency and fidelity may be obtained compared with normal PCR polymerase [2].

The detection limits of parasitemia in the standard PCR were estimated to be 0.00059% and 0.0002% with observation of the 202-bp ('C. M. haemominutum') and 273-bp (*M. haemofelis*) DNA fragments, respectively [15]. Furthermore, in a real-time PCR system, the efficiency of amplification of 'C. M. haemominutum' was in the range of 10⁷ starting templates (corresponding to 0.0000001% parasitemia) and that of *M. haemofelis* was in the range of 10⁶ starting templates (corresponding to 0.000001% parasitemia) [14]. Although the direct PCR was slightly less sensitive than the standard PCR and was rather less sensitive than real-time PCR, it may be sensitive enough for practical laboratory use.

Direct PCR may reduce the time required, cost and amount of technical work; moreover, it may be useful in avoiding contamination and confusion of samples. In the present study, we demonstrated that direct PCR using whole blood is a sensitive, rapid and convenient method to make a definitive diagnosis of *M. haemofelis* and 'C. M. haemominutum' infections in cats.

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